ANTIBIOTIC SUBSTANCES PRODUCED BY ACTINOMYCETES

BY

PRABHAT KUMAR BHATTACHARYYA

B.S., University of Calcutta, 1941
M.S., University of Calcutta, 1943

AN ABSTRACT OF A THESIS

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Introduction

The discovery, from time to time, of new antibiotics produced by actinomyces with remarkable chemotherapeutic properties extends an invitation for research in this field. Three antibiotic producing strains, 8-44, 8-96 and 9-20, all belonging to the Streptomyces species, isolated by Dr. David Gottlieb in the Horticulture Field Laboratory, University of Illinois, were found to produce the antibiotic materials in shake cultures in good yields in suitable media. These antibiotics were stable and were active against a wide range of microörganisms. It was the purpose of this investigation to isolate and characterize these antibiotics. The work is divided into three parts:

Part 1. Isolation of the active principle from the culture media of 8-44.

Part 2. Isolation and fractionation of the antibiotics from 8-96 cultures.

Part 3. Isolation of antibiotics from 9-20 culture media and physicochemical and degradation studies on one of them.

Discussion Of Results

Part 1.

Preliminary studies indicated that the antibiotic from 8-44 was neutral in nature, soluble in ether and butanol, and insoluble in petroleum ether, and chloroform. It was adsorbed on carbon and eluted by organic solvents. A process of extraction with ether was developed to remove the antibiotic from the culture medium. The crude ether extract was partly soluble in benzene and the benzene insoluble fraction con-

tained most of the activity. The benzene-insoluble fraction was recrystallized from a mixture of benzene-methanol to a light brown crystalline solid. Carbon adsorption and chromatography over alumina yielded an almost colorless crystalline product from the benzene insoluble residue. The final product after recrystallization from a mixture of chloroform-methanol, assayed 630 units per mg., melted at 146-148.5° and represented a 62 per cent recovery of the total activity in the culture medium. The antibiotic crystallized in thin long prisms and contained nitrogen and non-ionic chlorine. It was found to have identical properties with the Parke-Davis antibiotic, Chloromycetin. (1)

Part 2.

The strain 8-96 elaborated in its culture medium a mixture of basic antibiotics. The active principles were obtained as a crude extract by carbon adsorption, elution with methanolic hydrochloric acid and precipitation with ether. The crude material was converted to a mixture of *p*-hydroxyazobenzene-*p'*-sulfonate salts which was fractionated by stepwise recrystallization from water, and alcohol and converted to the hydrochloride by treatment with the exchange resin IR-4B in the chloride phase. Products with potency varying from 54 to 250 units per mg. were obtained by this procedure, from a crude material assaying 100 units per mg. By chromatography over alumina an amorphous, colorless and hygroscopic material assaying 260-310 units per mg. was obtained from a crude preparation assaying 100 units per mg. The purified antibiotic still contained at least two different materials, which were separated by the fractional recrystallization of the helianthates and p-hydroxy azobenzene-p'-sulfonates. One of the helianthates (m.p. 208-214°d.) resembled actinorubin helianthate (2) in analysis, one other fraction approached streptothricin helianthate in antibiotic potency and melting point (222°d.)

The mold 8-96 strongly resembled the actinorubin producing mold, *Actinomyces* A 105 in its cultural characteristics and

antibiotic spectrum.

Part 3.

Preliminary studies on the culture media of 9-20 revealed the existence of at least three different antibiotics; — (a) a basic antibiotic with antibacterial properties (b) an ether soluble antibiotic with both antifungal and antibiotic properties and (c) an ether insoluble material with both antifungal and antibacterial properties.

The basic antibiotic was isolated by adsorption on carbon, elution with acid-methanol, precipitation with ether and chromatography over alumina to an amorphous product assaying 100-125 streptomycin units per mg. and representing a 15 per cent recovery of the active material. The product gave positive ninhydrin and Molisch tests and negative Sakaguchi, Millon, maltol, Benedict and Hopkins-Cole tests, and therefore was different from either streptomycin or streptothricin.

The ether soluble antibiotic from 9-20 was concentrated by extraction by ether, a countercurrent distribution between *n*-hexane and 95 percent aqueous methanol, extraction with acetonitrile and chromatography of the acetonitrile extracts. The final product was a viscous oil assaying 1250 units per mg. and the yield was 50 per cent of the original activity. It had identical antibiotic spectrum with the ether insoluble factor from 9-20.

The ether insoluble antibiotic 9-20 was extracted with *n*-butanol from the brew and either with alcohol or with *n*-butanol from the mycelium. The extracts obtained by evaporation of the solvent were washed with ether, dissolved in water and precipitated with acetic acid. The active floculent gummy precipitate was reprecipitated once more from alkaline solution with acid and twice from alocholic solution with ether. The ether-precipitated material assayed 750- 1500 units per mg. and accounted for about three-fourths of the total activity. Recrystallizations from dioxane gave a semicrystalline residue

assaying 1250 units per mg.

The purified active material had a neutral equivalent of 3750 and gave positive reactions for the steroid nucleus. In the U. V. and infra-red absorption spectra and in some of the reactions the existence of conjugated double bonds or a phenyl group hydroxyl and carboxyl groups was indicated. Hydrogenation, acetylation and benzoylation of the purified antibiotic gave rise to products with little or no activity. Diazomethane treatment gave a semicrystalline product with a high activity. Periodate broke down the free acid antibiotic into a mixture of volatile and non-volatile aldehydes and acids of which acetaldehyde has been characterized through its 2-4,dinitrophenyl hydrazone. Nitric acid oxidation degraded the antibiotic into an amorphous acid containing nitrogen and with a neutral equivalent of 268. Acid hydrolysis gave a product which was alkali insoluble and which responded to the test for bile acids.

The antibiotic bore a striking resemblance to one other antibiotic, musarin (3). But it differed from the latter by its superior stability, the sterol reactions and the activity of the diazomethane reaction product. The antibiotic has been named endomycin.

Summary

A method has been developed for the isolation of the antibiotic from the culture media *Streptomyces* 8-44. This consists of extraction with ether, washing with benzene, crystallization from a mixture of benzene and methanol, chromatography over alumina, and crystallization from a mixture of chloroform and methanol. The antibiotic has been found to be identical with Chloromycetin.

A method has been developed for the isolation and partial fractionation of the antibiotics produced by *Streptomyces* 8-96 through carbon adsorption, chromatography and fractional recrystallization of the helianthates and *p*-hydroxyazobenzene-*p'*-sulfonates. One of the factors closely resembles

actinorubin and one other factor exhibits some similarity with streptothricin.

An isolation procedure for a new antibiotic, endomycin, has been developed. It consists of extraction of the antibiotic from the culture media and mycelia of *Streptomyces* 9-20 with *n*-butanol, precipitation of the active material as a free acid from aqueous solution, reprecipitation by ether from alcoholic solution, and fractionation with dioxane. The antibiotic is a high molecular weight hydroxy acid containing nitrogen, a phenyl group, and possibly a conjugated structure similar to that of a steroid. It gives an inactive hydrogenation product, inactive acetate, slightly active benzoate, and a highly active diazomethane reaction product. It is oxidized by permanganate, periodate, and nitric acid and destroyed by mineral acids. The periodate reaction mixture consists of a number of volatile and non-volatile aldehydes and acids of which acetaldehyde has been characterized. Endomycin is a relatively non-toxic antibiotic having strong antifungal and antibacterial properties.

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VITA

The author was born on October 22, 1921 in Calcutta, India. He passed the Matriculation Examination of the University of Calcutta in the year 1936 from Bagerhat H. E. School, Bagerhat, W. Bengal, India. He entered the Presidency College, Calcutta in July 1936 and received his Bachelor of Science Degree with Honours in Chemistry in 1941 from the University of Calcutta. From August, 1940 he studied as a Graduate student in the Department of Applied Chemistry, University of Calcutta and received his Master of Science degree in the year 1943. He was engaged as a research scholar in the same department from September, 1942 to December, 1946. He entered the Graduate College of the University of Illinois in February, 1947. In the department of chemistry he held a research assistantship for two and a half semesters and a third year graduate fellowship for two semesters.